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Albumin and α-Fetoprotein Gene Transcription in Rat Hepatoma Cell Lines Is Correlated with Specific DNA Hypomethylation and Altered Chromatin Structure in the 5′ Region†

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We examined DNA methylation and DNase I hypersensitivity of the α-fetoprotein (AFP) and albumin gene region in hepatoma cell lines which showed drastic differences in the level of expression of these genes. We assayed for methylation of the CCGG sequences by using the restriction enzyme isoschizomers *Hpa*II and *Msp*I. We found two methylation sites located in the 5′ region of the AFP gene and one in exon 1 of the albumin gene for which hypomethylation is correlated with gene expression. Another such site, located about 4,000 base pairs upstream from the AFP gene, seems to be correlated with the tissue specificity of the cells. DNase I-hypersensitive sites were mapped by using the indirect end-labeling technique with cloned genomic DNA probes. Three tissue-specific DNase I-hypersensitive sites were mapped in the 5′ flanking region of the AFP gene when this gene was transcribed. Similarly, three tissue-specific DNase I-hypersensitive sites were detected upstream from the albumin gene in producing cell lines. In both cases, the most distal sites were maintained after cessation of gene activity and appear to be correlated with the potential expression of the gene. Interestingly, specific methylation sites are localized in the same DNA region as DNase I hypersensitive sites. This suggests that specific alterations of chromatin structure and changes in methylation pattern occur in specific critical regulatory regions upstream from the albumin and AFP genes in rat hepatoma cell lines.

Many studies with different experimental systems have been carried out to elucidate the role that methylation of cytosine bases and change in chromatin structure might play in the regulation of gene expression (for reviews, see references 34 and 35).

Controversial results concerning the relationship between the DNA methylation pattern and gene activity have been reported showing either a good correlation (23, 47) or no correlation at all (13, 46, 57) between undermethylation and expression of a specific gene. Various studies involving in vitro methylation of a specific regulatory region of a gene followed by its introduction into eucaryotic cells revealed that DNA methylation does affect transcription (6, 17, 41, 42). Recent studies indicate that undermethylation of only certain critical sites located in the 5′ region of genes coding for tissue-specific proteins is necessary but not sufficient for their expression (24, 30, 56). These observations suggest that methylation stabilizes, rather than causes, gene expression.

Active genes have an altered chromatin structure that renders them highly sensitive to digestion by the nuclease DNase I (53; reviewed in reference 35). Recently, in addition to these DNase I sensitivity domains, short regions of DNase I hypersensitivity usually located 5′ to active or potentially active genes have been found (59; reviewed in reference 10).

Conflicting data have been reported concerning the hypomethylation of critical sites and the concomitant acquisition of DNase I-hypersensitive (DNase I HS) regions (39, 54; reviewed in reference 35). Therefore we have undertaken to examine the relationship between DNA methylation, the presence of DNase I HS sites, and the expression of the α-fetoprotein (AFP) and albumin genes, two markers of hepatic differentiation (1, 32), in rat hepatoma cell lines differing in the expression of these genes. For this purpose, we used variant cells of the Reuber H35 rat hepatoma that show reduced sensitivity to glucocorticoids (49). Some of these variants, called differentiated, express most hepatic functions including albumin and AFP synthesis, while the dedifferentiated variants, obtained from the differentiated ones, have ceased to express these genes (48, 50). Of these variants, we chose to study an AFP- and albumin-producing variant (Fα-5) and an AFP- and albumin-nonproducing one (clone 2) in addition to the parental Faza 967 cells that synthesize albumin but not AFP. These closely related cell clones offer a valuable model for studying the regulation of AFP and albumin genes.

In this report, we show that transcription of the AFP and albumin genes in the cell lines analyzed is well correlated with the hypomethylation of some *Msp*I sites and with the presence of DNase I HS sites. All these sites are located in the 5′ region of these genes. Their regulatory significance is supported by their localization near regions defined as important for albumin and AFP gene control.

MATERIALS AND METHODS

Rat tissues and cell lines. Male rats of the Sprague-Dawley strain (Iffa-Credo, St. Germain sur l'Arbresle, France) were used for isolation of nuclei and high-molecular-weight DNA from adult liver cells.

The Faza 967 clone is a differentiated (albumin-producer), glucocorticoid-sensitive (growth sensitivity) descendant of line H411EC3, derived from the Reuber H35 rat hepatoma (8, 36). Dexamethasone-resistant DEX-Faza 967 cells were derived from Faza 967 cells by using increasing concentra-

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† This article is dedicated to the memory of J. M. Sala-Trepat, who died while this work was in progress.

tions of dexamethasone. Clones Fα-5 and 2 were isolated from DEX-Faza 967 cells and were grown for about 8 months in the presence and then in the absence of dexamethasone. Fα-5 cells have been cultivated for about 1 year and express numerous liver-specific functions including albumin production (differentiated variant). AFP production was also detected in clone Fα-5, in contrast to the parental Faza 967 cells. Clone 2 cells were cultivated for several years and have lost the ability to express all liver-specific functions examined, including albumin and AFP (dedifferentiated variant). Details concerning the isolation, properties, and culture conditions of the variants are described elsewhere (48–51).

Hepatoma 7777-C8 is a clonal line derived from the Morris transplantable hepatoma 7777 originally induced in a Buffalo rat; its cells synthesize AFP but not albumin (47). The JF1 fibroblast subclone was derived from sarcoma cell line CCL45 (44); it does not express either the AFP or the albumin gene. Culture conditions are described elsewhere (47).

Preparation of nuclei. Nuclei from adult rat liver were isolated as described elsewhere (26).

Cells were scraped from 10 plates then collected by centrifugation for 5 min at 2,000 rpm. All subsequent steps were done in the cold room; the nuclei were maintained at 0 to 4°C. The pellet was suspended in 10 ml of homogenization buffer [15 mM Tris hydrochloride (pH 7.4), 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM spermidine, 0.15 mM spermine, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.34 M sucrose]. Then, Nonidet P-40 solution (final concentration, 0.2%) was added. Triton X-100 (final concentration, 0.2%) was also added for the Morris 7777-C8 and JF1 cell lines. The nuclei were homogenized gently by using a Dounce apparatus. They were pelleted and washed twice in homogenization buffer without detergent.

DNase I hypersensitivity analysis. The nuclei from rat liver and cell lines were suspended at a concentration of about 10^8 nuclei per ml in digestion buffer (15 mM Tris hydrochloride [pH 7.4], 60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.25 M sucrose). Nuclei were digested at 37°C for 2 min. We used 1 to 2 μg of DNase I (Worthington Diagnostics; 1,684 U per mg of enzyme) per ml for rat liver, Morris hepatoma 7777-C8, and JF1 clones. For Faza 967, Fα-5, and clone 2, we were obliged to use higher concentrations of DNase I (10 to 18 μg/ml) to yield the same level of digestion as for adult liver or other cell lines. Then the reaction was stopped, and the DNA was purified as described elsewhere (26). The genomic DNA (10 μg per sample) was digested with *Eco*RI (Boehringer GmbH) and then electrophoresed on 0.8 or 2% agarose gels.

DNA methylation analysis. The DNA from the Faza 967 Fα-5, and clone 2 cell lines was isolated from nuclei as described by Nahon et al. (26). High-molecular-weight DNA from the Morris 7777-C8 and JF1 cell lines and from rat liver cells was isolated by the method of Blin and Stafford (5).

Portions (10 μg) of DNA were digested with an excess (10 U/μg of DNA) of *Hin*dIII (Amersham Corp.) and *Eco*RI (Boehringer). DNA fragments were then digested with either *Msp*I (Pharmacia, Inc.) or *Hpa*II (Boehringer) added in excess (10 U/μg of DNA). The restriction fragments were separated on 1% agarose gels.

Southern transfer and hybridization procedures. Transfer of the separated fragments onto nitrocellulose paper (Schleicher and Schüll) or nylon membrane (Biodyne A; Pall Ultrafine Filtration Corp.) and hybridization with ^{32}P-labeled nick-translated DNA probes were performed essentially as described previously (21). Filter autoradiograms were quantitatively scanned with a Vernon densitometer.

Molecular hybridization probes. To obtain genomic subclones, the rat AFP genomic clones λRAFP15 and λRAFP22 (47) were digested with *Eco*RI and *Hin*dIII or *Eco*RI alone, respectively. The genomic probes pO_1, pO_2, pO_3, pO_4, pA_1, and pE are the restriction fragments obtained inserted in pBR325 (M. Poiret, M. Gomez-Garcia, and I. Tratner, unpublished results). We used probe pO_4 (1,390 nucleotides in length) to map DNase I HS sites upstream from the rat AFP gene (see Fig. 4C).

The genomic probes SubJA and SubJB (38) are double *Eco*RI-*Hin*dIII digestion fragments of the rat albumin genomic clone λRSA30 (37) inserted in pBR325. We used probe SubJB (1,450 nucleotides in length) to map DNase 1 HS sites upstream from the rat albumin gene (see Fig. 5C).

The purified plasmids were labeled by nick-translation as described by Maniatis et al. (22). The specific activity of the resulting recombinant [^{32}P]DNA was about 2×10^8 to 4×10^8 cpm/μg.

RESULTS

The various Reuber hepatoma cell lines that we have used provide different phenotypes for albumin and AFP gene expression. Indirect immunofluorescence staining for intracellular AFP and albumin and in situ hybridization studies showed that at the time of these experiments, most Faza 967 and Fα-5 cells (92 to 97%) produced albumin; 87% of the cells of clone Fα-5 were positive for AFP, while no AFP-positive cells were found in the parental Faza 967 population; and cells of the dedifferentiated clone 2 were uniformly negative for both proteins (Table 1). In vitro transcription assay and Northern blot analysis showed that the lack of production of the two serum proteins was the result of a transcriptional block (3, 7, 27), since the absence of detectable amounts of the corresponding mRNAs correlates with the cessation of transcription of the albumin and AFP genes (50; J.-L. Nahon and J. L. Danan, unpublished results).

Methylation analysis. To examine the methylation state of the albumin and AFP genes, we used the isoschizomer restriction endonucleases *Hpa*II and *Msp*I. Both enzymes recognize the sequence CCGG, but only *Msp*I will cut it when the internal cytosine is methylated.

Morris hepatoma 7777-C8 and adult liver cells were analyzed as positive controls for AFP and albumin gene expression, respectively. Indeed, it has been shown that all cells of the Morris hepatoma 7777-C8 cell line and all hepatocytes in the adult liver are engaged in AFP and albumin synthesis, respectively (4, 50). As the Morris hepatoma was originally induced in a Buffalo rat, it is not surprising to find a polymorphism for certain *Msp*I sites compared with Sprague-Dawley rats (see Fig. 2C, band ★). Such a polymorphism has already been reported for various restriction sites of rat albumin and AFP genes (11, 12). The JF1 fibroblastic cell line provides a complete negative control for both albumin and AFP gene expression. Moreover, it serves as an example of a nonhepatic cell line.

Methylation pattern of the AFP gene. We examined the methylation state of 10 *Msp*I sites in the various cell lines and in adult liver. Figure 1 shows the location of the sites on the AFP gene map, as well as the genomic subclones used to determine their level of methylation. Site M_0 maps in exon 1 about 30 nucleotides downstream from the TATA box (M.

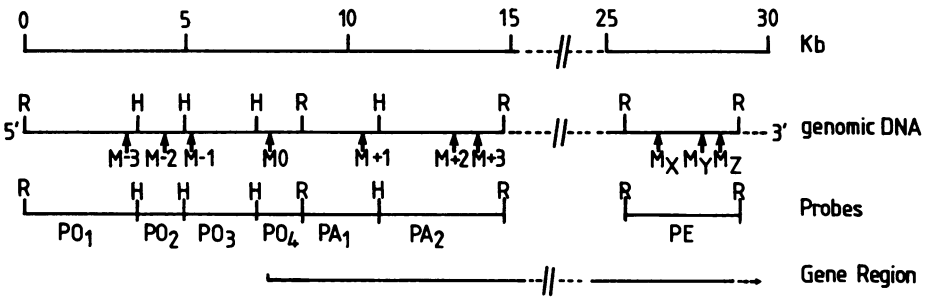

FIG. 1. Restriction map of the rat AFP gene. The location of *Msp*I-*Hpa*II sites was determined by double digestion of excised genomic subclones pO, pA, and pE. These subclones were obtained as described in Materials and Methods. Subcloning in pBR325 of pO and pA by *Hin*dIII digestion gave rise to pO1, pO2, pO3, pO4, pA1, and pA2, which are used as molecular probes in our experiments (M. Poiret, unpublished results). R, H, and M designate *Eco*RI, *Hin*dIII, and *Msp*I sites, respectively.

Poiret, unpublished results). M_{-1}, M_{-2}, and M_{-3} are located in the 5′ flanking region upstream from the M_0 site. M_{+1}, M_{+2}, and M_{+3} are positioned in the 5′ coding region, while M_X, M_Y and M_Z are around the 3′ end of the rat AFP gene.

Figure 2 shows some of the autoradiograms obtained with different AFP genomic probes. As each probe reveals the level of methylation of one unique site, only three bands are expected (numbered 1 to 3 in the figure). Bands 2 and 3 result from the cleavage of band 1 at the *Msp*I site probed. When *Hpa*II digestion is performed, the appearance of band 1 alone signifies that the probed site is fully methylated, the appearance of bands 2 and 3 signifies that the probed site is totally unmethylated, and the presence of all three bands indicates a partial methylation of the relevant site. The absence of the smaller fragment (band 3) from some of our autoradiograms is probably due to a reduced transfer efficiency for low-molecular-weight DNA fragments. This was the case for Fig. 2A and D.

Analysis of the methylation patterns shown in Fig. 2 indicates that sites M_0 and M_{-2} are mostly undermethylated in the AFP producer Fα-5 and Morris hepatoma 7777-C8 clones (Fig. 2B and C, lanes c and d), whereas the same sites are fully methylated in the AFP-nonproducer hepatic or nonhepatic cells (Fig. 2B and C, lanes a, b, e, and f). Moreover, the percentage of methylation of the M_0 site in the Fα-5 cell line (20%) is in good agreement with the percentage of AFP-nonproducing cells in this clone (Table 1). Therefore, the methylation of sites M_0 and M_{-2} seems to be well correlated with AFP gene expression. The demethylation of M_{-1} site might be a prerequisite for the cell line to be able to further express the AFP gene. Indeed, this site is demethylated in the parental Faza 967 cell line from which the AFP-producing Fα-5 clone was generated (50). In contrast, this site is fully methylated in the nonproducing clone 2. In contrast to the Faza 967 cell line, no reexpression of AFP was found in clone 2 after 2 years of dexamethasone treatment (50). This site is also fully methylated in the nonproducing JF1 cell line but is completely demethylated in the high-AFP-producing Morris hepatoma 7777-C8 line (Table 1).

Site M_{-3} shows a particular methylation pattern (Fig. 2A). This site appears to be undermethylated in all hepatic and hepatoma cells. On the contrary, it is highly methylated in the nonhepatic cell line JF1. This observation, along with results on DNase I sensitivity (see below), might indicate that the methylation state of this site is correlated with the tissue specificity.

No correlation with gene expression is found for the other sites examined within the structural AFP gene. This is illustrated by the analysis of site M_{+1} in Fig. 2D. Indeed, hypermethylation of this site is observed in Faza 967, clone 2, and Fα-5 cells independently of AFP gene expression. Similar results are obtained for sites M_{+2}, M_{+3}, M_X, M_Y and M_Z (data not shown).

Methylation pattern of the albumin gene. Three *Msp*I sites located in the 5′ region of the rat albumin gene were checked for their level of methylation in the various tissue and cell lines analyzed. Site M_{+1} maps about 80 base pairs downstream from the cap site in exon 1 of the rat albumin gene. Site M_{-2} and M_{-4} are located about 3,500 and 4,200 base pairs upstream from the cap site, respectively (Fig. 3C).

The albumin M_{+1} site is highly methylated in the albumin-nonproducing clone 2 (Fig. 3A, lane b), Morris hepatoma 7777-C8 (lane d), and JF1 (lane e) cells. The same site is

TABLE 1. Expression, methylation, and DNase I hypersensitivity patterns of albumin and AFP genes in the different cell lines

Cell type	AFP gene								Albumin gene						
	Expression (% of cells)	% Methylation[a]				Presence of DNase I HS sites[b]			Expression (% of cells)	% Methylation[a]			Presence of DNase I HS sites[b]		
		M_{-3}	M_{-2}	M_{-1}	M_0	AFPIII	AFPII	AFPI		M_{-4}	M_{-2}	M_{+1}	AlbIV	AlbII	AlbI
Faza 967	0[c]	0	100	0	100	++++	++	−	96[c]	>50	100	5	++++	++	+/−
Clone Fα-5	87[c]	0	10	0	20	++++	++++	+	94[c]	<40	100	<5	++++	++	+/−
Clone 2	0[c]	0	100	100	100	++++	−	−	0[c]	<30	100	100	++++	−	−
Morris 7777-C8	95[d]	0	10	0	0	++++	++++	+++	0[d]	100	−[e]	100	−	−	−
JF1	0[d]	90	100	100	90	−	−	−	0[d]	>40	<80	80	−	−	−

[a] From Fig. 2 and 3 and data not shown. The autoradiograms were scanned with a Vernon densitometer.
[b] From Fig. 4 and 5. The level of sensitivity to DNase I was estimated by scanning the DNase I-generated bands with a Vernon densitometer.
[c] From immunofluorescence staining experiments (50).
[d] From in situ hybridization experiments (A. Poliard, personal communication).
[e] −, See legend to fig. 3.

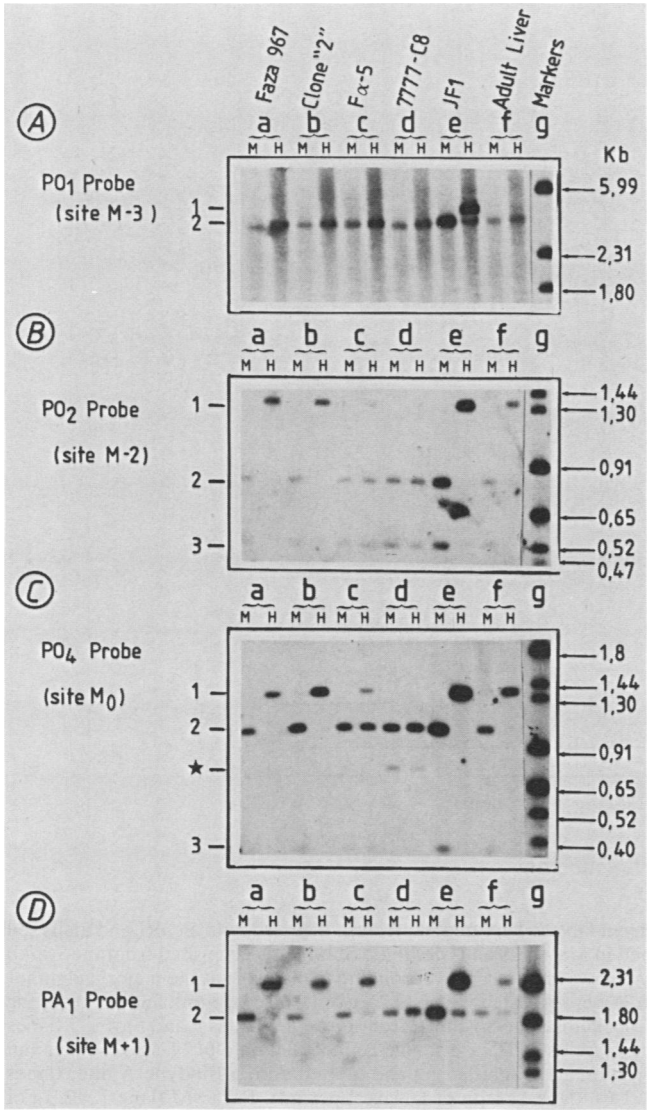

FIG. 2. Methylation state of the 5′ region of the rat AFP gene. Genomic DNA (10 μg) extracted from cell lines and tissue (lanes a to f) were digested by *Hin*dIII plus *Eco*RI and then either by *Hpa*II (H) or *Msp*I (M) as described in Materials and Methods. The restriction fragments were separated on a 1% agarose gel, transferred to a Biodyne A filter, and hybridized with AFP ^{32}P-labeled subclone pO1 (A), pO2 (B), pO4 (C), or pA1 (D). ★, Position of a 0.8-kb band present in the Morris hepatoma 7777-C8 lane and due to polymorphism in that DNA region between Buffalo and Sprague-Dawley rats (12). Molecular weight markers were run in lane g.

demethylated in the albumin-producing lines Faza 967 (lane a) and Fα-5 (lane c) and in adult liver (lane f). In all albumin-producing cell lines and tissues, the low percentage of methylation of the M_{+1} site strikingly reflects the small number of albumin-nonproducing cells present in the preparation (Table 1). We can therefore conclude that a good correlation exists between undermethylation of the M_{+1} site and transcription of the albumin gene. This result is in good agreement with previously published observations (29, 30).

Hybridization with probe SubJA results in a complex pattern due to both the presence of three MspI sites in that DNA region and their partial methylation (Fig. 3B). The estimation of percentages of methylation of M_{-2} and M_{-4}, summarized in Table 1, shows that the levels of methylation of these sites do not correlate with albumin gene expression. Indeed, except in adult liver, the M_{-2} site is mostly methylated even when the cell type analyzed transcribes the albumin gene. On the contrary, site M_{-4} is mostly demethylated (30 to 50%) in adult liver as well as in the cell lines except in the Morris hepatoma 7777-C8 line.

Pattern of DNase I HS sites upstream from the AFP gene. Nuclei were isolated from Faza 967, Fα-5, and clone 2 cell lines. As a control, nuclei were also isolated from the AFP-producing Morris hepatoma 7777-C8 cell line, the AFP-nonproducing fibroblast JF1 line, and adult liver (26). After DNase I digestion and Southern blotting, the DNA was hybridized with cloned genomic AFP probe pO4 (Fig. 4C), and the DNase I HS sites were mapped by the indirect end-labeling technique described by Wu (59).

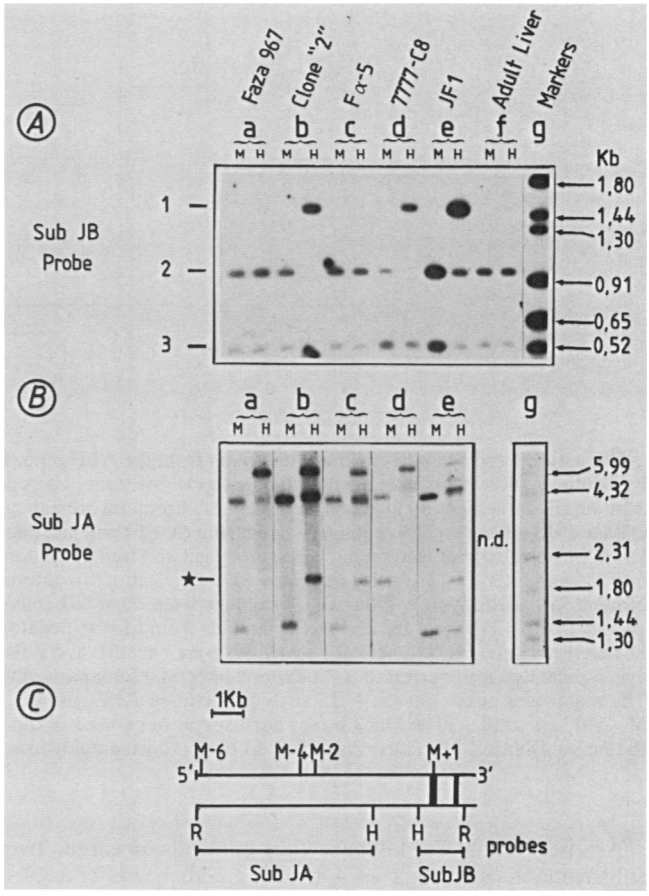

FIG. 3. Methylation state of the 5′ region of the rat albumin gene. Genomic DNA extracted from cell lines and tissue (lane a to f) was digested as described in the legend of Fig. 2. After separation on a 1% agarose gel and transfer to a filter, the restriction fragments were hybridized with ^{32}P-labeled albumin subclones SubJB (A) and SubJA (B). (C) Location of the *Msp*I sites (M) in the 5′ end of the rat albumin gene as described by Orlofsky and Chasin (29). R and H, *Eco*RI and *Hin*dIII restriction sites, respectively. Black vertical boxes designate exons; white boxes designate introns (38). ★, 1.9-kb band present in the *Msp*I digest of Morris hepatoma 7777-C8 cells, which replaces the 0.5-kb (not shown) and 1.4-kb bands present in other *Msp*I-digested samples. This could be due to either a methylation of the external cytosine of the M_{-2} site (*Msp*I cannot cut in such a case) or the absence of the M_{-2} site in the Buffalo DNA rat strain from which Morris hepatoma 7777-C8 cells were derived. In panel B, n.d. designates not determined.

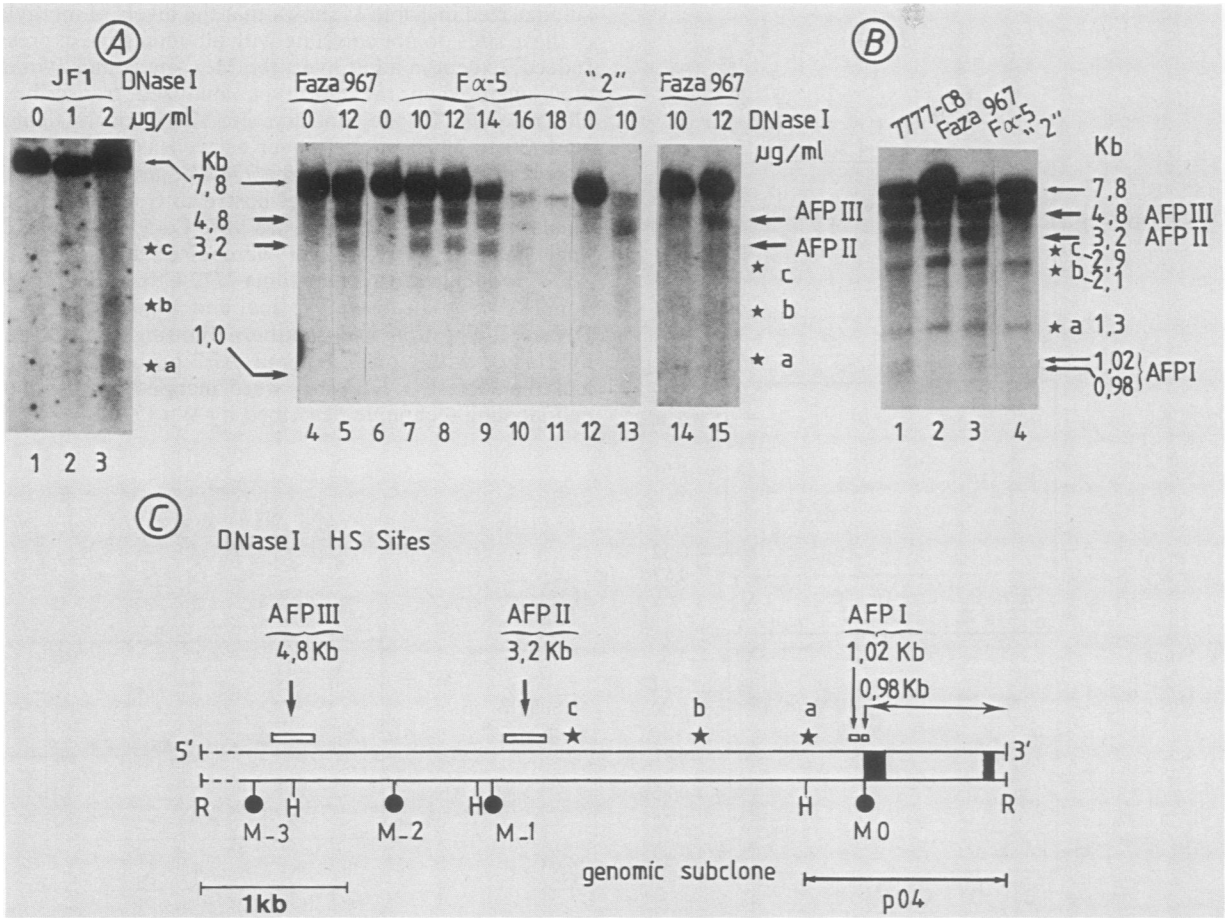

FIG. 4. DNase I hypersensitivity upstream from the AFP gene. DNA from DNase I-treated nuclei was digested with *Eco*RI and analyzed by Southern blotting by using genomic probe pO4 (see panel C) as described in Materials and Methods. Sub-bands generated from the 7.8-kb AFP original fragment by DNase I treatment of chromatin are indicated by arrows and stars corresponding to cleavage at the major and minor DNase I HS sites, respectively. (A) Nuclei from the JF1 cell line (lanes 1 to 3) were digested with 0 to 2 µg of DNase I per ml, and the resulting DNA was electrophoresed on a 0.8% agarose gel and transferred onto nitrocellulose paper; nuclei from Faza 967 (lanes 4 and 5), Fα-5 (lanes 6 to 11) and 2 (lanes 12 and 13) cell lines were digested with various concentrations of DNase I, electrophoresed on a 0.8% agarose gel, and blotted onto a Biodyne A filter. DNA samples from Faza 967 cells were run on a 1% agarose gel and blotted onto a Biodyne A filter (lanes 14 and 15). (B) DNase I-treated DNA samples from Morris hepatoma 7777-C8 (lane 1; 1 µg of DNase I per ml), Faza 967 (lane 2; 12 µg of DNase I per ml), Fα-5 (lane 3; 12 µg of DNase I per ml), and 2 (lane 4; 12 µg of DNase I per ml) cell lines were electrophoresed on a 2% agarose gel and transferred to a Biodyne A filter. (C) Summary of the DNase I HS sites and methylation sites upstream from the AFP gene. The major and minor DNase I HS sites are denoted AFPI, AFPII, and AFPIII and a, b, and c, respectively; the methylation sites are M_0, M_{-1}, M_{-2}, and M_{-3}. The black boxes correspond to exons 1 of the albumin gene. The white horizontal bars indicate the regions covered by the major DNase I HS sites. As indicated by the horizontal arrow, the distances were noted from the downstream *Eco*RI site.

As the original 7.8-kilobase (kb) band disappeared, two subfragments migrating as 4.8- and 3.2-kb bands (Fig. 4A and B) and a faint 1.0-kb band were revealed in the AFP-prducing Fα-5 cell line (Fig. 4B). This pattern is similar to that observed for the hepatoma 7777-C8 cell line (Fig. 4B), which expresses high level of AFP mRNAs (Table 1), except that the hybridization signal of the 1.0-kb band is drastically reduced (Fig. 4A). With a highly resolving gel (Fig. 4B), a doublet of 0.98- and 1.02-kb bands corresponding to the AFPI site was found in Morris hepatoma 7777-C8 cells (lane 1) and, although less intense, in Fα-5 cells (lane 3). In the parental Faza 967 clone and dedifferentiated clone 2 cells, which do not produce AFP (Table 1), the AFPI site is not visible, although 4.8-kb band corresponding to the most distal site (AFPIII) is fairly visible (Fig. 4A, lanes 5 and 13; Fig. 4B, lanes 2 and 4). Moreover, a 3.2-kb band corresponding to the AFPII site is also visible in the Faza 967 cells (Fig. 4A, lanes 5, 14, and 15). None of these sites was detected in the JF1 fibroblastic cell line (Fig. 4A, lanes 1 to 3). Additional DNase I HS sites are visible with a resolving 2% agarose gel. These sites, a, b, and c, are considered minor sites, as they are found in various rat tissues and cell lines without any correlation with gene expression (Fig. 4A and B; J. L. Nahon, A. Venetianer, and J. M. Sala-Trepat, Proc. Natl. Acad. Sci. USA, in press).

The AFPI site covers the promoter region of the AFP gene and part of exon 1 including methylation site M_0 (Fig. 4C). The AFPII site is located about 2.2 kb upstream from the promoter region of the AFP gene near methylation site M_{-1}. The distal AFPIII site lies close to methylation site M_{-3} at about 3.8 kb from the promoter region of the AFP gene.

Pattern of DNase I HS sites upstream from the albumin gene. We have mapped the DNase I HS sites upstream from the albumin gene by using the indirect end-labeling tech-

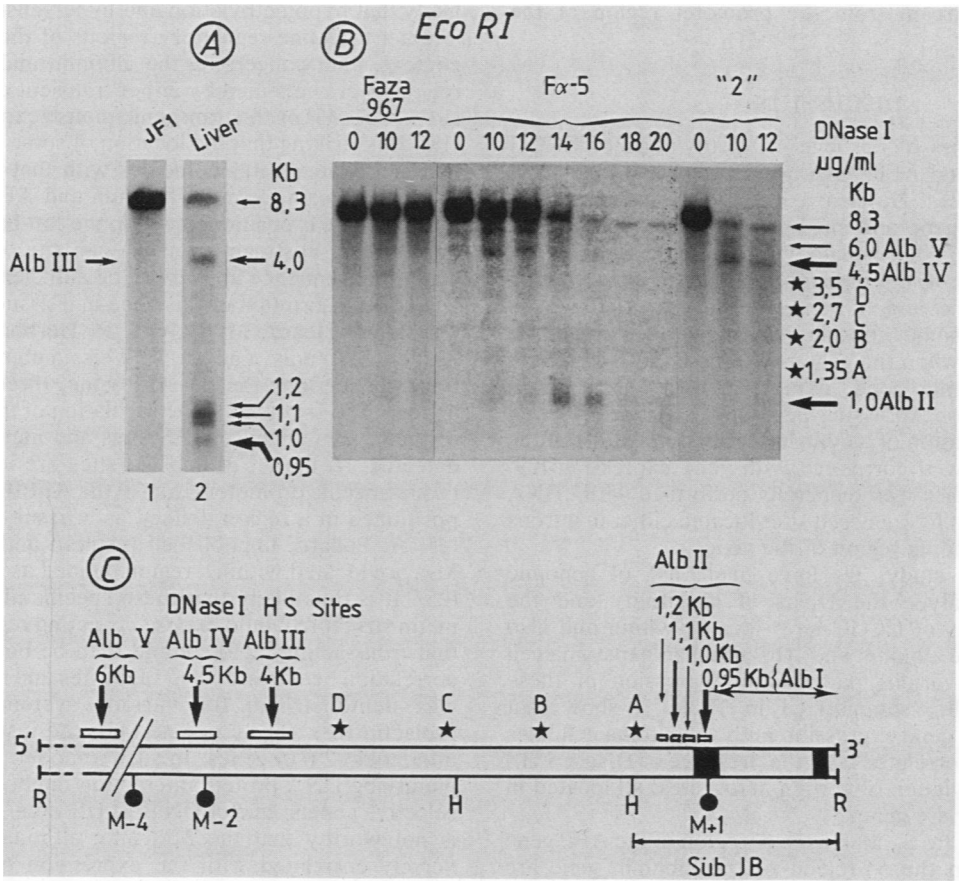

FIG. 5. DNase I hypersensitivity upstream from the albumin gene. DNA from DNase I-treated nuclei was digested with *Eco*RI and analyzed by Southern blotting as described in Materials and Methods. Filters were probed with subclone SubJB (see panel C). Sub-bands generated from the 8.3-kb albumin original fragment by DNase I treatment of chromatin are indicated by arrows and stars, corresponding to cleavage at the major and minor sites, respectively. (A) lane 1: JF1 cell nuclei digested with 2 µg of DNase I per ml; lane 2: adult liver nuclei digested with 2 µg of DNase I per ml. (B) Faza 967 and clone 2 nuclei were digested with 10 to 12 µg of DNase I per ml. Fα-5 nuclei were digested with 10 to 20 µg of DNase I per ml. (C) Summary of the DNase I HS sites and methylation sites upstream from the rat albumin gene. The major DNase I HS sites are denoted AlbI, AlbII, AlbIII, AlbIV, and AlbV; the minor DNase I HS sites are denoted A, B, C, and D. The methylation sites are M_{+1}, M_{-2}, and M_{-4}. R, *Eco*RI; H, *Hin*dIII. The significance of the horizontal bars, black boxes, and horizontal arrow is described in the legend to Fig. 4.

nique (59) with the cloned DNA genomic probe SubJB (Fig. 5C).

In adult liver chromatin, DNase I digestion of the original 8.3-kb band, corresponding to the 5′ flanking sequence of the rat albumin gene (Fig. 5C), generated three main subfragments migrating as a 4.0-kb band, a broad fragment centered at 1.1 kb, and a somewhat diffuse subfragment of 0.95 kb (Fig. 5A). These subfragments indicate the presence of three DNase I HS sites named AlbIII, AlbII, and AlbI in the 5′ region of the albumin gene in adult rat liver. Close inspection of the blot in the region corresponding to the AlbII site revealed the existence of three discrete bands of 1.0, 1.1, and 1.2 kb (Nahon et al., in press). None of these major DNase I HS sites was found upstream from the albumin gene in fibroblastic JF1 cell lines (Fig. 5A).

In DNA from albumin-producing hepatoma cells (Faza 967 and Fα-5 clones), DNase I-generated subfragments of 4.5 kb (AlbIV site) and a diffuse band centered at 1.0 kb (AlbII site) were present, while the 0.95-kb band (AlbI site) was hardly detected even with the higher concentrations of DNase I (Fig. 5B). Interestingly, we detected only the 4.5-kb band (AlbIV site) in the dedifferentiated clone 2 cells, which do not synthesize albumin (Fig. 5B). One additional subfragment migrating as a 6.0-kb band (AlbV site), which was not visible in adult liver (Fig. 5A), was visualized in the three hepatoma cell lines (Fig. 5B). Faint bands corresponding to sites AlbIV and AlbV were also detected in nuclei from the Fα-5 clone incubated without DNase I (Fig. 5B, Fα-5 lane 0), suggesting that the chromatin structure of these DNA regions renders them highly accessible to nucleases. Minor sites, named A, B, C, and D, were also detected in Reuber hepatoma-derived cell lines as in other rat tissues and cell lines (Fig. 5B; Nahon et al., in press).

It is noteworthy that the AlbIII and AlbIV sites appear to be specific for the tissue and cell lines, respectively. Indeed, the AlbIII site does not appear in any of the cell lines even after long exposure. Similarly, the AlbIV site stays undetectable in adult liver. This observation might indicate that slight differences exist between tissue and cell lines concerning the mechanisms that control the albumin gene expression.

Two of the major sites (AlbI and AlbII) are located within 0.5 kb of the 5′ end, close to methylation site M_{+1} (Fig. 5C). The AlbIV and AlbV sites are found about 3.5 and 5 kb,

respectively, upstream from the promoter region of the albumin gene.

DISCUSSION

Previous analyses of the level of methylation of CCGG sites of albumin and AFP genes were performed mainly by use of cDNA probes. No clear correlation between expression of the AFP gene and site-specific demethylation was found for fetal and adult hepatocytes and in AFP-producing oval cells (19, 33, 47). Comparison of an AFP-expressing hepatoma cell line and a nonexpressing one led to the conclusion that some specific intragenic sites might be undermethylated when the gene is expressed (20, 47). Concerning the albumin gene, parallel analysis of albumin-expressing and nonexpressing hepatoma cell lines showed that the demethylation of a cytosine located in exon 1 of the albumin gene is well correlated with gene expression (29, 30). As these studies were generally performed with cDNA probes, they might have missed sites located either in introns or in the 5′ noncoding region of the genes.

In the present study, we have made use of genomic subclones to analyze the DNase I sensitivity and the methylation status of CCGG sites located within and also upstream from the albumin and AFP genes in hepatoma cell lines that show various patterns of expression of these genes. Our results, summarized in Table 1, show that albumin and AFP gene expression in the hepatoma cell lines analyzed is well correlated with the presence of DNase I HS sites and demethylation of certain *Msp*I sites, all located in the 5′ region of these genes.

Two *Msp*I sites (M_{-2}, M_0) in the 5′ region of the AFP gene and one (M_{+1}) in the 5′ region of the albumin gene are hypomethylated when the gene is actively transcribed. Similarly, DNase I HS sites AFPI, AlbI, and AlbII are detected only when the corresponding genes are active. These DNase I HS sites, although all correlated with gene expression, are not all equivalent: their accessibility varies with the level of expression of the gene. The level of transcription of the AFP gene in Morris hepatoma 7777-C8 cells and of the albumin gene in adult rat liver is about 10-fold higher than that of the same gene in the expressing cell lines studied (26, 50). While the AlbII site shows high accessibility to DNase I in all albumin-expressing cell lines and tissues, AFPI and AlbI are increasingly accessible as the gene is more highly transcribed. Accordingly, AlbII might be considered an activating site, whose presence is associated with overt expression of the gene; AlbI would be a modulating site, whose accessibility is correlated with the level of transcription of the gene. AFPI seems to combine the two effects, since it is necessary for gene expression (as shown by using Faza 967 cells) and also shows variable accessibility depending on the level of gene expression. AFPII, present in cells that express AFP but also in Faza 967 cells, which do not, is probably necessary but not sufficient by itself to induce gene activity.

Of particular interest is the close localization of some *Msp*I and DNase I HS sites. Indeed, for at least three sites, a demethylated CG is positioned in a region of hypersensitivity to DNase I (AlbI and M_{+1}; AFPI and M_0; AFPII and M_{-1}). A fourth such couple is constituted by the AlbII site positioned over an *Ava*I site whose level of methylation is well correlated with albumin gene expression in rat hepatoma cell variants (M_{-1} site in reference 25). For both AFP and albumin genes, some of these couples are located in or near the promoter region; the AFPII-M_{-1} couple is positioned far upstream (about 2.5 kb). Therefore, it is very likely that hypomethylation and hypersensitivity to DNase I interact to define regulatory regions of these genes. In this context, data concerning the albumin and AFP regulatory regions were collected by either transient expression assays (14, 25, 31, 55) or the transgenic mouse experimental system (18). It is striking that the location of some DNase I HS sites and methylation sites coincides with that of the regulatory region upstream from the albumin and AFP genes. Indeed, the AlbII site is positioned within the 150-base-pair sequence immediately upstream from the cap site of the rat albumin gene; this sequence appears to be sufficient to direct a high level of transcription of this gene in a tissue-specific manner (31; J. M. Heard, M. O. Ott, P. Herbomel, A. Mottura-Rollier, M. Yaniv, and M. C. Weiss, submitted for publication). Concerning the rat AFP gene, three distinct regions appear to be involved in the regulation of the gene, and in all of them, some DNase I HS sites and methylation sites are detected: (i) the AFPI and M_0 sites are located within the tissue-specific promoter (25); (ii) the AFPII and M_{-1} sites are positioned in a region defined as a tissue-specific enhancer (25; A. Pollard, unpublished results); and (iii) AFPIII and M_{-3} are located within a region defined as a specific silencer (25). It is thus likely that these specific alterations in chromatin structure would expose *cis*-acting regulatory region so that *trans*-acting factors could then be bound (9, 60). Such correlation between DNase I HS sites and regulatory regions was demonstrated for various systems including the prolactin (28), the lysozyme (45), the insulin (52), and the interleukin-2 (40) genes. In such a model, demethylation by modifying DNA-protein interaction might interfere with the selective appearance of DNase I HS sites. In that context, it is noteworthy that the M_{-1} site, although not strictly inversely correlated with the expression of the AFP gene, appears to be well correlated with the appearance of the AFPII site (the nearest DNase I HS site). Indeed, M_{-1} is completely demethylated when the AFPII site is present and is fully methylated when the AFPII site is undetectable (Table 1). Consistent with an interacting relationship between methylation and chromatin conformation is the observation that complete CpG methylation of M13 α-actin constructs before transfer into mouse L cells prevents the formation of DNase I HS sites, although these sites are present when unmethylated constructs are used (16). Further support for this model comes from experiments showing that 5-azacytidine treatment, known to inhibit DNA methylation, provokes the appearance of DNase I HS sites accompanying transcription of the *ev*-1 locus of avian endogenous retroviral DNA in chicken cells (15).

By analyzing methylation (29; reviewed in reference 34) and chromatin conformation (2, 35, 58), some authors were able to propose that expressed genes might be organized in active domains characterized by their particular sensitivity to DNase I or their general undermethylation compared with that of nonexpressed genes. In this context, the AlbIV site is at the limit of the albumin gene methylation domain as it was defined by Orlofsky and Chasin (29) for rat hepatoma cell lines. Similarly, sites AFPIII and M_{-3} are in the region of the rat genome which corresponds to that defined as necessary and sufficient for AFP tissue-specific expression in the mouse (14). It is noteworthy that these sites are not correlated with the actual transcription of the genes but rather with the potentiality of the cells to express them. The absence of AlbIV from Morris hepatoma 7777-C8 cells would then suggest that these cells have lost the potentiality to express the albumin gene. Indeed, experiments performed on the Morris hepatoma 7777-C8 cell line to detect general

sensitivity to DNase I show an almost completely closed chromatin structure at the albumin locus (26). Therefore we propose that the particular conformation of these sites located at the upstream limit of the transcriptional domains of AFP and albumin genes maintains the gene in a pre-active state which facilitates downstream conformational changes within that domain. Such a role in the determination of the structural state of a chromatin domain has been proposed for methylation sites located within the transition region of DNase I sensitivity, at the downstream limit of the ovalbumin gene domain (43).

In conclusion, we suggest that undermethylation of specific CpG sites and the selective appearance of DNase I HS sites located close to these methylation sites are not fortuitous events but rather are correlated with various regulatory mechanisms leading to albumin and AFP gene transcription in the hepatoma cell lines analyzed. Most probably, the positions of these sites correspond to sequences involved in interactions with specific regulatory proteins.

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